Applied and Environmental Microbiology, Dec. 2008, p. 7174–7182 Vol. 74, No. 23
0099-2240/08/$08.00+0 doi:10.1128/AEM.01298-08

Quantification of Diatom and Dinoflagellate Biomasses in Coastal Marine Seawater Samples by Real-Time PCR▽

Anna Godhe,[1]* Maria E. Asplund,[1] Karolina Härnström,[1] V. Saravanan,[2] Anuj Tyagi,[2] and Indrani Karunasagar[2]

Department of Marine Ecology, University of Gothenburg, Box 461, SE 405 30 Göteborg, Sweden,[1] and Department of Fishery Microbiology, College of Fisheries, Karnataka Veterinary Animal and Fisheries Sciences University, P.B. No. 527, Mangalore 575 002, India[2]

Received 11 June 2008/Accepted 2 October 2008

Two real-time PCR assays targeting the small-subunit (SSU) ribosomal DNA (rDNA) were designed to assess the proportional biomass of diatoms and dinoflagellates in marine coastal water. The reverse primer for the diatom assay was designed to be class specific, and the dinoflagellate-specific reverse primer was obtained from the literature. For both targets, we used universal eukaryotic SSU rDNA forward primers. Specificity was confirmed by using a BLAST search and by amplification of cultures of various phytoplankton taxa. Reaction conditions were optimized for each primer set with linearized plasmids from cloned SSU rDNA fragments. The number of SSU rDNA copies per cell was estimated for six species of diatoms and nine species of dinoflagellates; these were significantly correlated to the biovolumes of the cells. Nineteen field samples were collected along the Swedish west coast and subjected to the two real-time PCR assays. The linear regression of the proportion of SSU rDNA copies of dinoflagellate and diatom origin versus the proportion of dinoflagellate and diatom biovolumes or biomass per liter was significant. For diatoms, linear regression of the number of SSU rDNA copies versus biovolume or biomass per liter was significant, but no such significant correlation was detected in the field samples for dinoflagellates. The method described will be useful for estimating the proportion of dinoflagellate versus diatom biovolume or biomass and the absolute diatom biovolume or biomass in various aquatic disciplines.

Several diverse taxa are represented in the phytoplankton communities of coastal marine environments. These contribute to primary production and form the base of the marine food chain. Annual variation in primary production is caused by external factors such as nutrient access, light, and temperature (18). Seasonal patterns—including changes in phytoplankton diversity, community composition, and biovolumes—also affect the magnitude of primary production. Additionally, short-term fluctuations within the phytoplankton community are common and are due to the dynamics of local hydrographic conditions (1), zooplankton grazing (48), and exchange between sediment and the water column (6, 27). Thus, the structure of the phytoplankton community is influenced by several biotic and abiotic factors, and the taxonomic composition will, in turn, affect other functions of the marine ecosystem.

Two of the most prominent and important phytoplankton classes in coastal marine waters and freshwater bodies are diatoms, Bacillariophyceae, and dinoflagellates, Dinophyceae. The taxonomic class Bacillariophyceae comprises approximately 100,000 extant species; planktonic species are predominantly autotrophic, and cell sizes range from 2 μm to 5 mm (37). The number of dinoflagellate species in the marine phytoplankton is approximately 2,000, of which 50% are heterotrophic and the rest are auto- or mixotrophic. Their cell sizes range from 2 μm to 2 mm (45). The variability within these classes is vast in many respects, but genetic, physiologic, and morphological features are common to all of the species of the respective classes.

Ecological and environmental studies often rely on accurate estimation of the density of a particular phytoplankton class or the ratios between the different taxonomic classes. For instance, dinoflagellates are suggested to promote the survival and dispersal of pathogenic marine bacteria by releasing bioavailable dissolved organic substances (9); conversely, many diatom genera are known to have an adverse effect on the growth of the same bacteria (33). Zooplankton feeding behavior is known to be modified according to the type of prey available (5). Diatoms are the favored prey for copepods and are rich in polyunsaturated fatty acids, which in turn is correlated with high egg production rates (32), but the proportional egg hatching success is frequently lower with a diatom-based diet. Dinoflagellates, on the other hand, are ingested at a much lower rate and consequently the egg production rate is decreased, but in compensation, there is increased egg hatching success (5). Shellfish which have become noxious due to the ingestion of toxin-producing microalgae exhibit faster biodepuration if they feed on diatoms rather than on nontoxic dinoflagellates (21); this is particularly important for the aquaculture industry. Diatoms and dinoflagellates succeed one another along hydrographic gradients, such that centric diatoms are generally more abundant at low sea surface temperatures (SST) and high NO_3^- concentrations, whereas dinoflagellates are favored by higher SST and lower NO_3^- concentrations (19). Thus, a future increase in global SST would probably disadvantage diatoms as a class (15).

Consequently, various aquatic disciplines have an interest in

* Corresponding author. Mailing address: Department of Marine Ecology, University of Gothenburg, Box 461, SE 405 30 Göteborg, Sweden. Phone: 46 31 7862709. Fax: 46 31 786 2727. E-mail: anna.godhe@marecol.gu.se.

▽ Published ahead of print on 10 October 2008.

quickly and accurately assessing the biomass of members of these classes at different spatial and temporal scales. Normally, this type of monitoring involves microscopic examination of phytoplankton samples. In many studies, data on cell abundances are inadequate due to the large size differences within the phytoplankton classes (29, 42). By recording cell sizes, cell abundances can be transformed into the much more appropriate biomass. This is time-consuming and requires considerable taxonomic experience, first because the identification is based on subtle distinctions between minute morphological characteristics and second because the width, length, and height of several individual cells of each species have to be measured. An adequate method for rapid and specific quantification would therefore be of great interest. Currently, there are, as far as we are aware, no available molecular techniques to quantify the total abundance of diatoms. Class-specific primers targeting the small-subunit (SSU) ribosomal DNA (rDNA) and their application in a conventional PCR assay have been developed for dinoflagellates (22), but there is no molecular-level method to quantify the total abundance of dinoflagellates in natural field samples. We have chosen to develop real-time PCR protocols to assess the proportional biomass of diatoms and dinoflagellates in field samples. Real-time PCR is advantageous because of its linearity and sensitivity and the speed with which a large number of samples can be processed. Real-time PCR protocols are currently available for other phytoplankton classes, i.e., for Chlorophyta (50) and Haptophyta (17). In addition, the numbers of protocols targeting individual dinoflagellate species (e.g., see references 4, 11, 25, and 31) or diatom species (7) and their application in environmental microbiology are rapidly expanding.

In this paper, we describe two new primer sets to be used in real-time PCR assays for the quantification of diatoms and dinoflagellates from marine field samples. The primers were initially tested on cultures, and the number of rDNA copies per cell was estimated for several species of each algal class. Finally, the two protocols were applied to numerous samples from the field. We selected the months of May and June for the collection of samples from the Swedish west coast. During these months, the succession of phytoplankton taxa is temporally and spatially dynamic and the proportion of dinoflagellates and diatoms may fluctuate rapidly (24). Thus, this suite of field samples permitted us to maximize our examination of the power of the tests for specificity and accuracy.

MATERIALS AND METHODS

Cultures. Several cultures were chosen for use in the various stages of our work, i.e., to test primer specificity, to serve as templates for the optimization of the real-time PCRs, and to estimate the number of rDNA copies per cell (Table 1). The majority of the strains are maintained at the University of Gothenburg Marine Algal Culture Collection (GUMACC) (www.marecol.gu.se/gumacc.html). Aliquots of algal strains in the early exponential growth phase were preserved in Lugol's iodine (45). These cells were settled into 10-ml sedimentation chambers overnight (47) or onto Sedgewick Rafter chambers (26), and abundances (numbers of cells per milliliter) were estimated by counting a minimum of 300 cells in an inverted microscope at ×400 or ×1,000 resolution. The enumeration technique was chosen according to cell size and the density of the culture. The lengths and widths of 10 random individual cells from different cell chains, if applicable, in each culture were measured. Biovolumes were calculated according to Sun and Liu (43). Simultaneously, algal cells were harvested from 20 ml of each of the cultures for DNA extraction by mild centrifugation (5,000 × *g*) for 5 min. Pellets were resuspended in 420 μl Milli-Q water and transferred to Microfuge tubes. Cells were lysed, and DNA was extracted by phenol-chloroform extraction as described previously (13). After extraction, DNA concentration (micrograms per milliliter) and purity were measured with a spectrophotometer (GeneQuant II; Pharmacia Biotech, Buckinghamshire, United Kingdom) or a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

TABLE 1. Algal strains used in this study

Species	Class	No. of GUMACC strains	Source[a]
Target species			
Lingulodinium polyedrum	Dinophyceae	112	CCMP1738
Heterocapsa triquetra	Dinophyceae	71	KAC86
Scrippsiella trochoidea	Dinophyceae	110	CCMP1331
Protoceratium reticulatum	Dinophyceae	109	CCMP1889
Prorocentrum micans	Dinophyceae	35	CCMP1589
Pentapharsodinium faeroense	Dinophyceae	87	K-0499
Prorocentrum lima	Dinophyceae	72	CCMP685
Gymnodinium chlorophorum	Dinophyceae	99	K-0539
Alexandrium andersonii	Dinophyceae	92	JL24
Skeletonema marinoi	Bacillariophyceae	143	
Ditylum brightwellii	Bacillariophyceae	73	
Coscinodiscus granii	Bacillariophyceae	140	CCAP1013
Cylindrotheca closterium	Bacillariophyceae	125	CCMP1554
Phaeodactylum tricornicum	Bacillariophyceae	2	UTEX 642
Extubocellulus spinifer	Bacillariophyceae	68	
Nontarget species			
Anabaena lemmermannii	Cyanophyceae		KAC16
Aphanizomenon cf. *klebahnii*	Cyanophyceae		KAC15
Nodularia spumigena	Cyanophyceae		KAC10
Rhodomonas sp.	Cryptophyceae	9	CCMP758
Emiliania huxleyii	Haptophyceae	47	
Prymnesium parvum	Haptophyceae	37	KAC16
Heterosigma akashiwo	Raphidophyceae	120	K-0541
Fibrocapsa japonica	Raphidophyceae	116	K-0542
Dinobryon balticum	Crysophyceae		CCMP1766
Dictyocha speculum	Dictyochophyceae		CCMP1381
Aureococcus anophagefferens	Pelagophyceae		CCMP1794
Tetraselmis sp.	Prasinophyceae	19	CCMP905
Dunaliella tertiolecta	Chlorophyceae	5	

[a] If not originally isolated at GUMACC. CCAP, Culture Collection of Algae and Protozoa; CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; KAC, Kalmar Algal Collection; K, SCCAP Scandinavian Culture Center for Algae and Protozoa; UTEX, The Culture Collection of Algae, The University of Texas at Austin; JL, Jacob Larsen, University of Copenhagen.

Field samples. Field samples were collected along the Swedish west coast during May and June 2007 (Table 2). Water samples from Kristineberg Marine Research Station (KMRS) were collected from the surface (0- to 3-m depth) with a plastic 1.6-liter Ruttner water bottle. Samples from other locations were collected with a flexible segmented hose (23). The content of the hose was emptied into a bucket, and the water from 0- to 10-m depths was mixed.

Lugol-fixed samples (23 to 50 ml) (45) were settled in 50-ml sedimentation chambers (47) overnight, and the phytoplankton community was characterized at ×200 and ×400 with a Zeiss Axiovert 135 inverted microscope. One subsample from each field sample was subjected to microscopic analysis. The number of cells counted and measured for biovolume estimation was dependent on the abundance of the target cells per liter of seawater. The minimum number of target cells counted was 97 in field sample 3, and the maximum was 949 in field sample 14. Species identification was based on the reports of Thomsen (44), Throndsen et al. (45), and Tomas (46) and the Checklist of Phytoplankton in the Skagerrak-Kattegat (available at www.smhi.se). The dimensions of all recorded dinoflagellate and diatom taxa were measured, and biovolumes were calculated by using formulae for the geometric shapes closely approximating the taxa (43). Cellular carbon content was calculated from biovolumes, by class-specific carbon volume conversion equations (29). From the same samples, known volumes of seawater (100 to 500 ml) were filtered through a 3-μm (diameter, 25 mm) cellulose membrane filter at low vacuum (100 mm Hg). The filters were loosely folded and put in Microfuge tubes with 500 μl lysis buffer (13). The tubes were vortexed and then held at 50°C for 1 h. Subsequently, the tubes were vortexed and then centrifuged at 4,000 × *g* for 3 min and the filter was transferred to a new

TABLE 2. Field samples collected in 2007 and analyzed in this study

Sample no.	Sampling date	Location	Vol of filtered seawater (ml)	No. of rDNA copies (10^9 liter^{-1})[a]		No. of cells (10^3 liter^{-1})		Biovolume (mm^3 liter^{-1})		Cellular carbon (μg liter^{-1})	
				Diatoms	Dinoflagellates	Diatoms	Dinoflagellates	Diatoms	Dinoflagellates	Diatoms	Dinoflagellates
1	8 May	KMRS	100	0.18 (0.08)	1.38 (0.03)	36	14	0.009	0.042	0.12	1.32
2	14 May	Bökevik	300	1.64 (0.30)	0.33 (0.03)	23	71	0.025	0.103	0.28	2.77
3	14 May	KMRS	300	0.49 (0.14)	0.97 (0.16)	25	25	0.018	0.049	0.22	1.52
4	15 May	Hakefjord	300	0.88 (0.34)	6.04 (0.66)	67	100	0.032	0.172	0.35	4.23
5	15 May	Stillingsön	200	0.10 (0.03)	0.29 (0.04)	19	114	0.007	0.261	0.10	5.94
6	15 May	Koljöfjord	300	0.03 (ND)	0.07 (0.02)	11	84	0.005	0.293	0.07	6.53
7	16 May	KMRS	300	0.87 (0.44)	0.77 (0.09)	11	53	0.058	0.203	0.57	4.84
8	22 May	KMRS	400	0.21 (0.05)	0.75 (0.12)	114	119	0.049	0.124	0.50	3.23
9	24 May	KMRS	500	0.31 (0.12)	1.28 (0.32)	275	143	0.191	0.529	1.50	10.60
10	30 May	KMRS	400	1.92 (0.75)	0.47 (0.07)	117	307	0.268	0.295	1.97	6.56
11	4 June	Kosterfjord	400	0.48 (0.08)	0.64 (0.12)	39	32	0.108	0.177	0.94	4.33
12	4 June	Brofjord	400	0.72 (0.28)	1.46 (0.13)	201	204	0.546	0.163	3.51	4.04
13	5 June	Koljöfjord	400	0.32 (0.12)	1.16 (0.29)	53	143	0.096	0.333	0.86	7.25
14	5 June	Danafjord	400	1.42 (0.41)	1.45 (0.03)	1,254	59	1.019	0.075	5.82	2.15
15	5 June	Havstensfjord	400	0.46 (0.20)	0.60 (0.07)	330	25	0.075	0.251	0.70	5.75
16	5 June	Åstol	400	0.74 (0.23)	0.69 (0.13)	604	69	1.546	0.092	8.16	2.53
17	11 June	KMRS	300	5.02 (1.92)	3.24 (0.56)	218	187	3.732	0.848	16.68	15.60
18	19 June	KMRS	400	14.75 (1.30)	0.25 (0.02)	801	101	2.378	0.220	11.57	5.17
19	25 June	KMRS	400	11.10 (1.66)	0.14 (0.03)	777	106	3.934	0.214	17.41	5.05

[a] Values in parentheses are standard deviations. ND, no data.

Microfuge tube with lysis buffer. The procedure was repeated, and thereafter the filter was discarded. The contents of the two tubes per sample were pooled, and DNA was extracted as described for the cultures.

Primer development. Approximately 50 SSU rDNA sequences from GenBank for each target class, Bacillariophyceae or Dinophyceae, were downloaded from http://www.ncbi.nlm.nih.gov. The downloaded sequences were mainly from planktonic taxa. The sequences were thereafter grouped by class and aligned in Sequencher v.4.1.2. (Gene Codes Corporation, Ann Arbor, MI). In aligning the Bacillariophyceae sequences, we included sequences representing the SSU rDNA from other, phylogenetically related, Heterokontophyta, i.e., Dictyophyceae, Pelagophyceae, Eustigmatophyceae, Chrysophyceae, Bolidophyceae, Pinguiophyceae, and Raphidophyceae. From the alignments, locations displaying minimal intraclass variation but maximal interclass variation were defined. Primers for the detection of diatoms were designed with Primer3 (39). The potential primer sites were further tested by computer-based analysis against databases with BLASTN 2.2.15 (2; all GenBank, EMBL, DDBJ, and PDB sequences) and Fasta (34; all EMBL sequences).

Primer specificity analysis. Potential primer pairs for the quantification and identification of Bacillariophyceae and Dinophyceae were tested with cultured target and nontarget species (Table 1). The annealing temperature was gradually increased to attain maximum specificity. For the diatom assay, annealing temperatures in the range of 57 to 60°C were tested, and for the dinoflagellate assay, temperatures in the range of 60 to 65°C were tested. The PCRs were run in a total volume of 25 μl each, consisting of approximately 50 ng template DNA and *Taq* buffer containing 1.5 mM $MgCl_2$, 0.5 μM each primer, 200 μM each deoxyribonucleotide triphosphate, 2.5 U of *Taq* polymerase (Qiagen, Valencia, CA), and sterile Milli-Q water. Amplifications were carried out with a thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Wellesley, MA) as follows: initially, 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57 to 65°C for 1 min, and extension at 72°C for 1 min. After the cycles, extension was completed at 72°C for 5 min. The PCR products were loaded onto a 2% agarose gel in 1× Tris-borate-EDTA, and ethidium-bromide-stained gels were studied under UV transillumination.

Preparation of plasmid standards and optimization of the real-time PCR assays. The partial SSU rDNA gene from extracted *Skeletonema marinoi* (GUMACC 143) DNA was amplified with universal ribosomal gene primers 1055F (10) and 1517R (49) under the same conditions as for the primer specificity analysis, with the exception of the annealing temperature, which was set to 60°C. The SSU rDNA gene from extracted *Prorocentrum lima* (GUMACC 72) DNA was amplified with eukaryotic SSU rDNA-specific primers (28). The same PCR conditions as for the primer specificity analysis were applied, but the annealing temperature was set to 50°C and the number of cycles was extended to 35. The PCR products were loaded onto a 0.8% agarose ethidium bromide-stained gel in 1× Tris-borate-EDTA, and the success of the reaction was confirmed after UV transillumination. The amplified diatom and dinoflagellate SSU rDNA fragments were cloned into TOPO vectors and transformed into competent *Escherichia coli* by following the protocol of the TA cloning system (Invitrogen). Plasmids were extracted with a Qiagen Plasmid Purification kit by following the protocol of the manufacturer (Qiagen). Linearized plasmids were produced after digestion with BamHI (New England BioLabs). The concentrations of the linearized plasmids were measured spectrophotometrically. The number of copies in the diatom and dinoflagellate plasmid standards was calculated by using a formula provided by Zhu et al. (50).

Reaction conditions for the real-time PCR assays were optimized for the two primer sets by using the plasmid standards as the template. In order to obtain the highest amplification efficiency of the real-time PCR assays, different primer concentrations were tested in the range 200 to 900 nM.

Real-time PCR analysis of monoclonal cultures. Real-time PCR assays were performed on 15 monoclonal cultures (six diatom strains, nine dinoflagellate strains) in order to estimate the number of rDNA copies per cell and the relationship between biovolume and gene copy number. To quantify the number of plasmid-rDNA fragment equivalents in the DNA extracts from the cultures, the plasmid standards were serially diluted in five 10-fold dilutions. Extracted DNA (17.5 to 186.7 ng μl^{-1}) from cultures with known densities of cells was diluted with sterile Milli-Q water in order to encompass the large density variation between cultures and to minimize PCR inhibition due to remaining culturing medium, cellular contents, or lysis detergents (5×10^{-2}, 10^{-3}, 5×10^{-3}, 10^{-4}, 5×10^{-4}, and 10^{-5} for diatom strains and 10^{-2}, 5×10^{-2}, 10^{-3}, 5×10^{-3}, 10^{-4}, 5×10^{-4}, and 10^{-5} for dinoflagellate strains). A minimum of three dilutions were used to calculate the number of rDNA copies per microliter of DNA extract. The final reaction mixture volume for all samples was 25 μl, and it contained 12.5 μl Sybr green PCR master mix (2×; Applied Biosystems, Warrington, United Kingdom), 1 μl of DNA extract, 300 nM each primer in the diatom assay, and 400 nM each primer in the dinoflagellate assay. A reaction mixture of PCR master mix and primers (24 μl) was loaded in triplicate into wells (white, 96-well, Thermo-Fast detection plate; ABgene), followed by the diluted DNA extracts (1 μl). Sterile Milli-Q water was used in three reaction mixtures as nontemplate controls. The plate was closed with QPCR optically clear adhesive seals (ABgene) and centrifuged for 2 min at 3,000 rpm to remove bubbles. Reactions were performed in an Applied Biosystems 7300 Real-Time PCR System cycler with separate programs for the two phytoplankton assays. The diatom assay started with an initial soaking step at 50°C for 2 min and activation of AmpliTaq Gold DNA Polymerase (from the Sybr green PCR master mix) at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. To observe the melting curve, a final dissociation cycle (preprogrammed in the 7300 sequence detection system software from Applied Biosystems) was added (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). For the dinoflagellate assay, the soaking, activation, and dissociation

steps were the same as those described above for the diatoms but differed in the 40 cycles as follows: denaturation at 95°C for 15 s, annealing and extension at 65°C for 30 s, and finally a melting step at 76°C for 45 s to reduce disturbance due to primer-dimers. Data from the real-time PCR assays were analyzed with the 7300 sequence detection system software from Applied Biosystems.

Real-time PCR analysis of field samples. For each field sample, two separate real-time PCR assays were performed to estimate proportions between diatoms and dinoflagellates; these assays used the class-specific primers and plasmid standards containing the class-specific gene fragments. Five dilutions of the DNA extracts from the field samples (39 to 236 ng μl^{-1}) were prepared for each diatom assay (10^{-2}, 5×10^{-2}, 10^{-3}, 5×10^{-3}, and 10^{-4}), and six were prepared for each dinoflagellate assay (5×10^{-1}, 10^{-2}, 5×10^{-2}, 10^{-3}, 5×10^{-3}, and 10^{-4}). The real-time PCR assays were performed as described above to estimate the ratio between the two phytoplankton classes on the basis of the number of gene copies per liter of the original water sample.

Reproducibility of the analysis of the ratio of the target molecules in the field samples was estimated by filtering the same volume of seawater twice from two different field samples and thereafter extracting DNA in duplicate from each sample as described above. Subsequently, the two separate real-time PCR assays targeting diatoms and dinoflagellates were performed on each DNA extract. The reproducibility of the DNA extractions and the real-time PCR estimation of the ratio of dinoflagellate to diatom rDNA copies was assessed as follows: the mean and standard deviation of each replicated ratio ($n = 2$) was calculated. The coefficient of variation (CV) was calculated for each sample and reported as a percentage ($1 - CV \times 100$).

Calculations. To estimate the number of rDNA copies per genome, the plasmid standard with a known copy number per microliter was plotted against the number of rDNA copies per microliter of DNA extract and thereafter divided by the cell density representing 1 μl of DNA extract. Thus, the regression slope represented an estimate of the number of SSU rDNA copies per cell for each cultured strain. The gene copy number per cell was subsequently analyzed against the length and biovolume of the cells, in turn, to establish a relationship between cell size and number of gene copies for the different species of cultured cells.

The proportion of SSU rDNA copies per liter of seawater of the two phytoplankton classes was analyzed for significant correlations (chi-square tests and symmetric tests) with the proportion of dinoflagellate and diatom biovolumes and biomass. Similarly, the number of SSU rDNA copies per liter seawater was analyzed for a significant correlation with biovolumes, biomass, and the number of cells per liter of diatoms and dinoflagellates. Significant cross tabulations were subjected to linear regression analyses. Cross tabulation tests and linear regression analyses were made in SPSS 11.0.4 for Mac OS X (SPSS Inc., Chicago, IL).

RESULTS

Primer design and validation. For the diatom assay, we designed the class-specific reverse primer, but for the dinoflagellate assay, the specific reverse primer was obtained from the literature (22). For both targets, we used previously published eukaryotic ribosomal gene primers as forward primers. Primer 1209f (diatoms) and primer EUK528f (dinoflagellates) were selected to minimize primer-dimer formation, complementarity, and melting temperature (T_m) differences and to obtain proper amplicon sizes for the real-time PCR assays. Computer-based analysis of primer specificity with BLASTN 2.2.15 and Fasta indicated that the class-specific primer designed to target diatoms was highly specific; it differed from that of other Heterokontophyta (i.e., related taxa) by at least four base pairs. SSU rDNA fragments from a range of species (Table 1) were amplified by the specific primers (Table 3); these amplified fragments were of the expected sizes (189 bp for diatoms, 110 bp for dinoflagellates). On the other hand, these primers did not yield any PCR product with the negative controls, representing a wide range of species that were at a greater taxonomic distance (Table 1).

Optimization of the real-time PCR assays. Reaction conditions for the real-time PCR assays were optimized for the two primer sets. The optimal primer concentrations were determined to be 300 nM for the diatom assay and 400 nM for the dinoflagellate assay. In the real-time PCR assay that targeted dinoflagellates, primer-dimer formation was observed and a final melting step at 76°C for 45 s was included. For both of the assays, the T_m of the amplicon was higher than that for the primer-dimer by 5°C or more.

TABLE 3. Primers used in this study

Target and primer	Sequence	T_m (°C)	Reference
Dinophyceae			
EUK528f	5′ CCGCGGTAATTCCA GCTC	57	10
Dino18SR1	5′ GAGCCAGATRCDCA CCCA	59	22
Bacillariophyceae			
1209f	5′ CAGGTCTGTGATGC CCTT	54	12
Diatom18SR1	5′ CAATGCAGWTTGATG AWCTG	50	This study

The two standard curves consisted of 10-fold serial dilutions of the plasmid SSU rDNA sequence from *Prorocentrum lima* for the dinoflagellate assay and the partial SSU rDNA sequence of *S. marinoi* for the diatom assay (10^8 to 10^2 copies for the dinoflagellate-containing plasmids and 10^7 to 10^3 copies for the diatom-containing plasmids). The plasmid standard curves showed a significant linear relationship between the threshold cycle (C_T) values and the initial number of plasmid copies (dinoflagellates, $R^2 = 0.987$, standard error [SE] = ±0.004, $n = 14$; diatoms, $R^2 = 0.989$, SE = ±0.002, $n = 14$). The efficiency of the real-time PCR, which was estimated by the equation $E = 10^{(1/k)} - 1$, where k is the slope of the standard curve, varied from 65 to 107% for the dinoflagellate assay and from 73 to 101% for the diatom assays. In order to validate the results from the field samples and the culture extracts, only those dilutions that showed the same linear relationship between C_T values and the initial gene copy number as the standard curve used in the same real-time PCR run were chosen. Consequently, in each real-time PCR run, at least three or four dilutions could be used to calculate the number of rDNA copies, which all were within the range of 9×10^5 to 1×10^2 for the dinoflagellates and 8×10^6 to 2×10^2 for the diatoms. For some field samples and culture extracts, the most concentrated dilutions showed higher C_T values than expected on the basis of the standard curves, indicating inhibition of the amplification reaction; thus, these dilutions were excluded from the estimations of the gene copy numbers. Accordingly, some of the most diluted samples were also excluded since they fell out of the range of the standard curve.

Real-time PCR of monoclonal cultures. The number of SSU rDNA molecules per cell ranged from 61 (*S. marinoi*) to 36,896 (*Ditylum brightwelli*) for the diatoms and from 1,057 (*Pentapharsodinium faeroense*) to 12,812 (*Lingulodinium polyedrum*) for the dinoflagellates. The number of gene copies per cell was significantly ($P < 0.001$) correlated with the biovolume of the cell (Fig. 1).

Field samples and real-time PCR. Field samples were collected from fjords along the Swedish west coast over approxi-

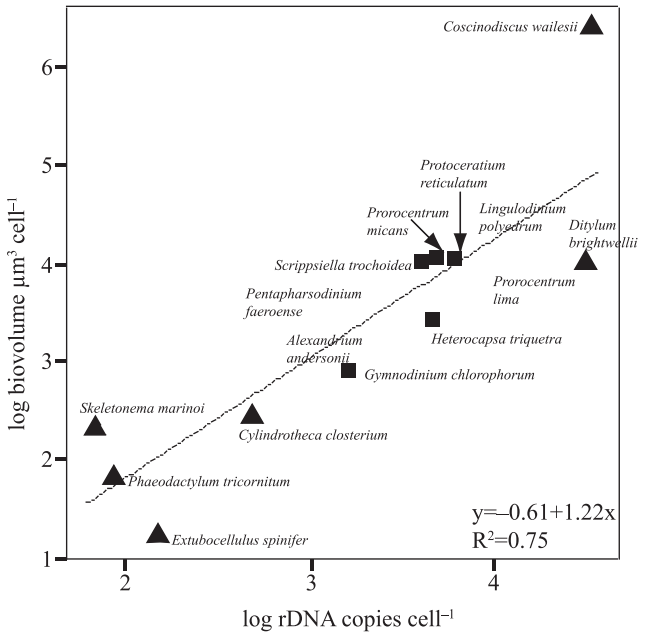

FIG. 1. Correlation between SSU rDNA copy number estimated by real-time PCR and cell biovolume of cultured algal strains. Symbols: ■, dinoflagellates; ▲, diatoms.

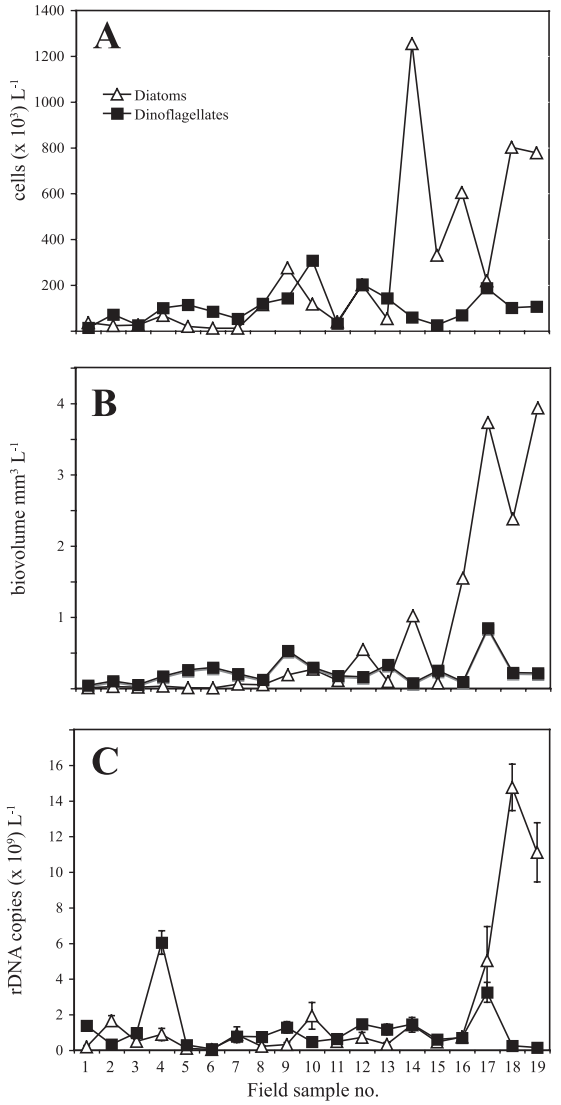

FIG. 2. (A) Cell abundances (10^3 liter^{-1}) of each target class, diatoms and dinoflagellates, in field samples collected during May and June 2007. (B) Biovolumes (cubic millimeters per liter). (C) rDNA copies (10^9 liter^{-1}). Error bars represent standard deviations.

mately 1.5 months (Table 2). In order to estimate the number of SSU rDNA copies, all field samples were subjected to two real-time PCR assays, one for diatoms and one for dinoflagellates. For both targets, the field samples were highly variable with respect to the number of SSU rDNA copies, cell abundance, biovolume, and cellular carbon per liter of seawater (Fig. 2A to C and Table 2).

In the early field samples (no. 1 to 7), the phytoplankton community was dominated by various small heterotrophic flagellates (e.g., *Telonema antarcticum*), ciliates, and low to moderate abundances of small (e.g., *Prorocentrum minimum*, Gymnodiniales) or heterotrophic (e.g., *Protoperidinium* sp.) dinoflagellates (14 to 114,000 cells liter^{-1}, Fig. 2A to C) and the diatoms encountered were mainly of benthic origin (e.g., *Paralia sulcata*, Pennales). By the end of May (field samples 8 to 10), the number of small heterotrophic flagellates had declined, the phytoplankton community was dominated by dinoflagellates (up to 300,000 cells liter^{-1}, Fig. 2A to C, e.g., *Ceratium tripos*, *Dinophysis norvegica*, *Prorocentrum minimum*, Gymnodiniales, *Protoperidinum depressum*), and the number of planktonic diatoms had increased (a maximum of 275,000 cells liter^{-1}, Fig. 2A). In June, the abundance of diatoms further increased (Fig. 2A to C). The latter two-thirds of June (field samples 17 to 19) revealed strong increases in the number of SSU rDNA copies of diatom origin ($<15 \times 10^9$ copies liter^{-1}, Fig. 2C), total diatom abundances (>800,000 cells liter^{-1}, Fig. 2A), biovolumes (<4 mm^3 liter^{-1}, Fig. 2B), and cellular carbon (11 to 16 mg C liter^{-1}, Table 2); these were due to a bloom of *Dactyliosolen fragilissimus* and *Proboscia alata*. Thus, the proportion of dinoflagellates was relatively high in the first 10 field samples, while the diatoms dominated the later samples (no. 11 to 19).

The extracted DNA from the field samples was diluted five times for the diatom assay and six times for the dinoflagellate assay in order to encompass the large proportional variation between the two target classes and to minimize PCR inhibition. The estimation of the number of rDNA copies per microliter of DNA extract for each target was based on three or four dilutions, with the exception of field samples 5 and 6 (Table 2). On these two occasions, the diatom abundances were comparatively low; i.e., cell abundances were 19,333 and 11,013 liter^{-1}, biovolumes were 0.007 and 0.005 mm^3 liter^{-1}, and cellular carbon contents were 0.10 and 0.07 mg liter^{-1}, respectively. Because of this, only the triplicate reactions from the 10^{-2} dilution of field sample 5 and only one reaction from the 10^{-2} dilution of field sample 6 could be used for the estimation of the number of diatom rDNA copies per liter of seawater. Thus, these two field samples were excluded from the regressions (ratio of dinoflagellate to diatom rDNA copies

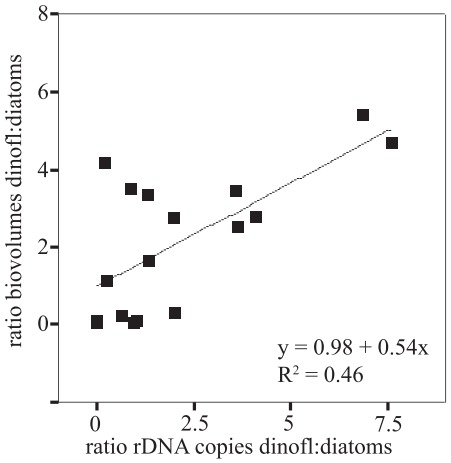

FIG. 3. Correlation between the ratio of dinoflagellate and diatom SSU rDNA copies and biovolumes per liter of seawater in field samples collected along the Swedish west coast. The linear regression was significant ($P < 0.01$). y is the ratio of dinoflagellate to diatom biovolumes, and x is the ratio of dinoflagellate to diatom SSU rDNA copies obtained by the two real-time PCR assays.

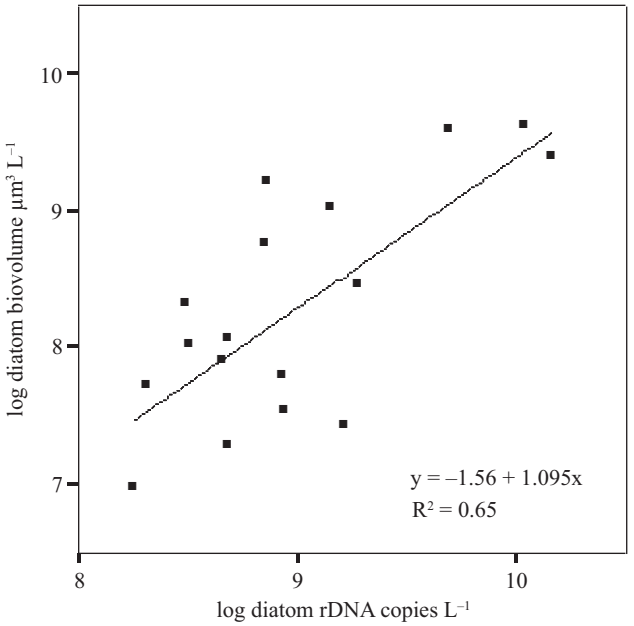

FIG. 4. Correlation between the number of diatom SSU rDNA copies and diatom biovolume per liter of seawater in field samples collected along the Swedish west coast. The linear regression was significant ($P < 0.001$). y is the log of the diatom biovolumes (cubic micrometers), and x is the log of the number of diatom SSU rDNA copies obtained by the real-time PCR assay.

versus biovolumes and diatom rDNA copies versus biovolumes or cellular carbon).

The linear regression of the proportion of SSU rDNA copies of dinoflagellate and diatom origin versus the proportion of dinoflagellate and diatom biovolumes per liter was significant ($P < 0.01$, Fig. 3). Cellular carbon of the respective class was based on cell volume estimation and was obtained through diatom- and dinoflagellate-specific carbon volume conversion equations. Hence, the linear regression of the proportion of dinoflagellate and diatom SSU rDNA copies versus the proportion of dinoflagellate and diatom cellular carbon content per liter was significant ($P < 0.01$). The relationship obtained was Ratio of dinoflagellate to diatom C = $2.75 + 1.18x$, where x is the ratio of the numbers of dinoflagellate and diatom SSU rDNA copies obtained from the two real-time PCR assays ($R^2 = 0.46$). The linear regression of the number of diatom SSU rDNA copies and diatom biovolumes per liter was significant ($P < 0.001$, Fig. 4). Similarly, the linear regression of the number of diatom SSU rDNA copies and diatom cellular carbon content per liter was significant ($P < 0.001$). The relationship obtained was log diatom C (picograms per liter) = $-1.879 + 0.895x$, where x is the log of the number of diatom rDNA copies per liter ($R^2 = 0.65$). No significant correlation was detected in the field samples between the number of dinoflagellate SSU rDNA copies and dinoflagellate biovolumes or cellular carbon content.

DNA was extracted in duplicate from the same volume of water from two different field samples (no. 2 and 3, Table 2), and from each DNA extract, the ratio of dinoflagellate to diatom SSU rDNA copies was estimated by the real-time PCR assays. The reproducibility of the DNA extraction and the real-time PCR assays for the ratio of dinoflagellate to diatom SSU rDNA copies was 78% (standard deviation, 5.7; $n = 2$).

DISCUSSION

Primer design and specificity. Initially, during the course of the primer design, genes other than the SSU rRNA were considered. For instance, the 5.8S rDNA and the *rbcL* gene, which codes for the large subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, were scrutinized. However, GenBank accessions of SSU rDNA far outnumber the available accessions for the other two genes, in terms of both target and nontarget accessions. Thus, the SSU rDNA was selected because the large number of target and nontarget accessions in GenBank provided an important source for initial primer specificity analysis. Because of the difficulty in finding sites that were both inclusive of target sequences and exclusive of nontarget sequences, many different combinations of primers had to be drafted and tested first by computer analysis and then by PCR on the target and nontarget DNA extracts of the cultures. The DNA extracts from monospecific cultures used as negative controls (and evaluated here in the primer specificity controls) represent only a small fraction of the SSU rDNA available in the environment. Nevertheless, the primer pair designed for diatoms was specific; this was revealed by primer specificity analysis in BLASTN and Fasta, by conventional PCR, and subsequently by a significant linear relationship between the number of SSU rDNA copies versus biovolumes and cellular carbon contents of diatoms from cultures and field samples. Likewise, the computer-based analysis and the conventional PCR (with DNA extracts from target and nontarget cultures) indicated high selectivity for the dinoflagellate-targeted primer pair.

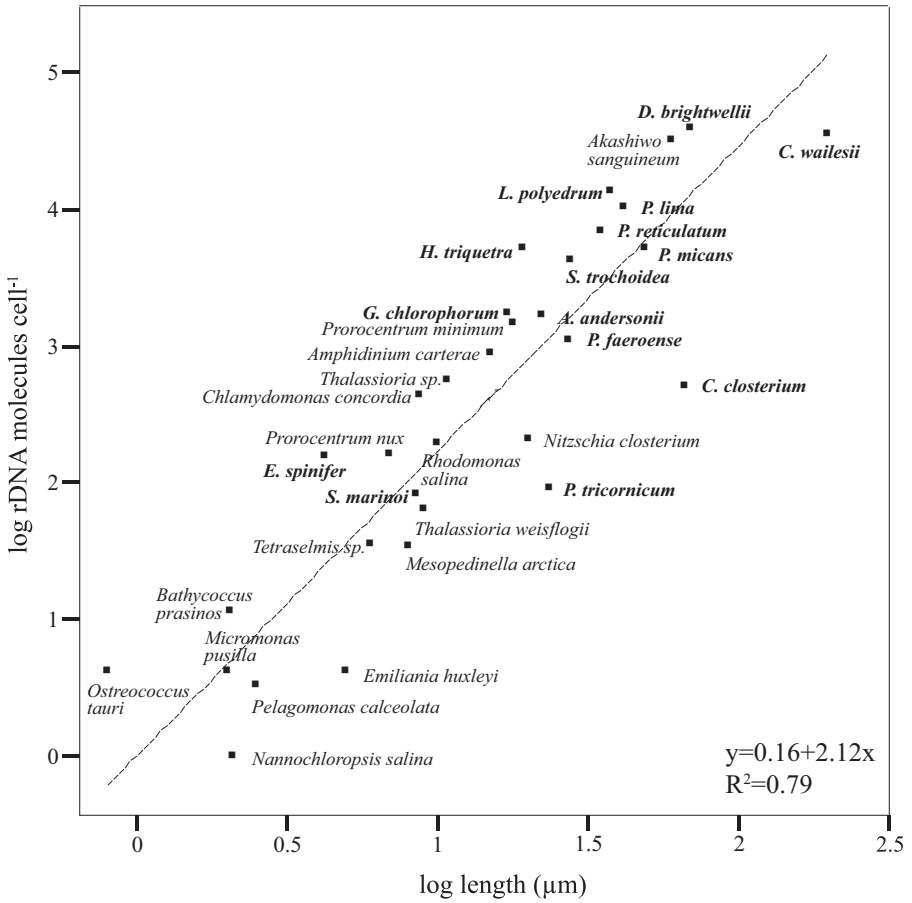

FIG. 5. Correlation between SSU rDNA copy number estimated by real-time PCR and cell length of cultured algal strains from this study and from that of Zhu et al. (50). Species analyzed in this study are in bold type.

Quantifying the number of rDNA copies in cultured cells. Previous studies have shown that the number of rDNA copies is related to genome size in eukaryotes, which in turn is related to cell size. Among 162 investigated species, the rDNA copy number varied in the range of 10 to $>10^4$ in animals and plants (35). More specifically, the relationship between rDNA copy number and cell length has also previously been confirmed for marine protists (50). We were able to verify this relationship with our data set, but we could also determine a linear relationship between the cell copy number and the biovolume, which is much more ecologically relevant. The number of rDNA copies in protists is significantly ($P < 0.001$) correlated with cell length (Fig. 5). The monoclonal culture of *Cylindrotheca closterium* (*Nitzschia closterium*) analyzed by Zhu et al. (50) had an average cell length of 20 μm and 199 rDNA copies per cell, whereas the monoclonal culture of *C. closterium* analyzed by us had an average cell length of 67 μm and 484 rDNA copies per cell. The cell size of this species is highly variable (45). Repeated vegetative division reduces diatom cell size, and size regeneration is generally thought to involve sexual reproduction (37). Thus, the culture with the larger cell size could have been harvested sooner after auxospore formation. This implies that smaller individual cells would have fewer gene copies compared to larger clonal counterparts, which would have yet to be demonstrated. A more plausible explanation would be that size variation, and subsequent variation in gene copy number, within strains of the same species may reflect the presence of genetically distinct clones. This is further supported by previous records, where genetically differentiated populations of diatoms have been shown to have different cell sizes (40, 41).

Detection limits and reproducibility of the assays. Environmental samples were diluted in two *n*-fold series, starting with a 50-fold dilution for the dinoflagellate assay and a 100-fold dilution for the diatom assay. More concentrated template DNA unambiguously impeded the real-time PCR. Inhibitory substances such as humic acids, metals, and polysaccharides are common in marine field samples (30), and dilution of the template DNA is a widely used way to circumvent these inhibitory effects (e.g., see reference 20). Moreover, the phenol-chloroform-based extraction protocol we used is known to effectively lyse and extract DNA from diatoms and dinoflagellates, but it sometimes yields suboptimal purity ($OD_{260} \div OD_{280} = <1.7$; A. Godhe et al., unpublished data), as has been recorded for many other organisms (e.g., see reference 38). Perhaps a different DNA extraction protocol could permit less dilution of the samples, and with this, the number of diatom molecules (as in field samples 5 and 6) might have been confidently calculated. However, extraction protocols may differ

widely in the yield of DNA obtained and could therefore affect the reproducibility of the assays.

Field sample 6 displayed a comparatively low number of dinoflagellate SSU rDNA copies per liter, but still we could use two dilutions (5×10^{-1} and 10^{-2}, each in triplicate) to estimate the copy number. In field samples 5 and 6, we could base the calculation of the number of diatom SSU rDNA copies on only one dilution, which is scientifically undesirable. Hence, the limit of accurate detection by the real-time PCR assay for diatoms is approximately 20 cells ml^{-1}, whereas the limit of detection by the dinoflagellate assay is lower. In comparing the dinoflagellate result of an unimpeded real-time PCR assay at a dilution of 5×10^{-1} with the diatom result wherein only the 10^{-2} dilution was usable, we propose that the cause of this disparity was probably the difference in the protocols. The higher annealing temperature and an additional extra melting step in the dinoflagellate protocol probably also served to control impurities accompanying the template DNA.

Real-time PCR allows very precise measurements of target DNA (3, 11) (Table 2), but the DNA extraction can add a substantial amount of variation to the total amount of DNA in a sample and seriously affect the quantification of the target molecules. Several factors, such as differences in proteinase activity, the amount of DNA retained in DNA extraction columns, or pipetting errors, can account for this variation. Primarily, the total amount of DNA in the sample is affected, while the relative amount of DNA of two target groups remains largely unchanged. Hence, the approach used here to estimate the proportion of two important coastal phytoplankton classes is advantageous not only from an ecological perspective but also from a technical point of view. The reproducibility of the ratio of dinoflagellate to diatom SSU rDNA copies was satisfying, 78%, and is comparable to previous studies (36).

Application of real-time PCR to field samples. In this study we have demonstrated a significant relationship between the proportion of rDNA copies and biovolumes of two dominant classes of phytoplankton in field samples, and, hence, the real-time PCR-based technique presented here can be used to quickly asses proportional biomass. Investigations of, e.g., phytoplankton grazing relationships, nutrient dynamics and turnover, and species- or class-specific contributions to primary production are commonly based on the labor-intensive activity of cell abundance and volume determination and subsequent transformation into biomasses. The weakness of this method is caused by errors in cell size measurements, which are cubed to give volumes, and the effect of fixatives, which can cause shrinking or swelling of cells. The relationships presented in this study are based on measurements of several Lugol-fixed individual cells of each species from the field samples collected, and constraints relating to cell measurements and fixatives apply to this procedure. Nevertheless, we feel this method is robust enough and will enable routine proportional biomass estimations for several pelagic applications, which are currently not possible.

No significant correlation was observed between the number of dinoflagellate rDNA copies and biovolumes or cellular carbon in the field samples, despite the significant linear regression of rDNA copies and biovolumes per cell for the nine dinoflagellate cultures. Theoretically, it should be easier to construct and apply class-specific primers for dinoflagellates than for diatoms because the dinoflagellates are phylogenetically more isolated (14). In this and previous (50) studies to evaluate the correlation between cell size and numbers of gene copies, the dinoflagellate cultures used as positive controls were all accustomed to a phototrophic habitat. In all of our field samples, obligate heterotrophic and mixotrophic dinoflagellate species were recorded and contributed significantly to the total recorded dinoflagellate biovolume and biomass. Heterotrophic dinoflagellates are nonselective feeders and have a complex feeding behavior, which also includes cannibalism (16). In the samples with proportionally high abundances of heterotrophic dinoflagellates, the correlation between the dinoflagellate number of gene copies and dinoflagellate biovolume (or biomass) was distorted. We therefore hypothesize that the nonlinearity of dinoflagellate gene copy number and biovolume (or biomass) in the field samples is due to the feeding behavior of the heterotrophic dinoflagellates. In these samples, dinoflagellate biovolumes were recorded by microscopy and the number of SSU rDNA copies from the dinoflagellate predators was determined by the real-time PCR assay. But the ingested dinoflagellate prey also contributed additional dinoflagellate SSU rDNA copies that were detected by the real-time PCR assay. In real-time PCR-based studies of copepod predation, the prey DNA was detectable in the guts of the predators for as long as 3 h (8). It is therefore reasonable to assume that nuclear DNA ingested by heterotrophic dinoflagellates will be retained long enough to be recorded by real-time PCR assays before it is finally digested.

Conclusion. In this study, we have designed and evaluated two primer sets for quantifying diatoms and dinoflagellates and their optimized real-time PCR protocols. We have addressed the significant relationship between cell biovolume and the number of rDNA copies per cell. Additionally, we have demonstrated the usefulness of this technique for estimating, in marine coastal field samples, the proportion of dinoflagellate versus diatom biovolume or biomass and the absolute diatom biovolume or biomass. This method constitutes a tool for quick and accurate monitoring the proportion of biovolume and biomass of two important phytoplankton taxa of coastal marine water.

ACKNOWLEDGMENTS

We are thankful to Ann-Sofi Rehnstam-Holm for her support in designing specific primers and to Antonella Penna for critically reading the manuscript. We are also thankful to Iddya Karunasagar for thoughtful advice on the design of the study. Odd Lindahl and Bodil Hernroth provided us with field samples through the plankton-monitoring group at KMRS. Three anonymous reviewers are acknowledged for their constructive comments on the manuscript.

This work was supported by grants from Sida (SWE-2006-022), Sida-Formas (2007-179), Swedish Research Links (2006-2462), the University of Gothenburg Marine Research Center (GMF), KVVS (Kungl. Vetenskaps- och Vitterhets-Samhället i Göteborg), and the Wilhelm and Martina Lundgrens Fund.

REFERENCES

1. **Almeida, M. A., M. A. Cunha, and F. Alcântara.** 2002. Seasonal change in the proportion of bacterial and phytoplankton production along a salinity gradient in a shallow estuary. Hydrobiologia **475:**251–262.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.

3. **Bennett, J. M., S. Kaye, N. Berry, and R. S. Tedder.** 1999. A quantitative PCR method for the assay of HIV-1 provirus load in peripheral blood mononuclear cells. J. Virol. Methods **83:**11–20.
4. **Bowers, H. A., T. Tengs, H. B. Glasgow, J. M. Burkholder, P. A. Rublee, and D. W. Oldach.** 2000. Development of real-time PCR assays for rapid detection of *Pfisteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol. **66:**4641–4648.
5. **Calliari, D., and P. Tiselius.** 2005. Feeding behaviour and reproduction in a small calanoid copepod: *Acartia clausi* can compensate quality with quantity. Mar. Ecol. Prog. Ser. **298:**241–250.
6. **Carstensen, J., P. Henriksen, and A.-S. Heiskanen.** 2007. Summer algal blooms in shallow estuaries: definition, mechanisms, and link to eutrophication. Limnol. Oceanogr. **52:**370–384.
7. **Creach, V., A. Ernst, K. Sabbe, B. Vanelslander, W. Vyverman, and L. J. Stal.** 2006. Using quantitative PCR to determine the distribution of a semicryptic benthic diatom, *Navicula phyllepta* (Bacillariophyceae). J. Phycol. **42:**1142–1154.
8. **Durbin, E. G., M. C. Casas, T. A. Rynearson, and D. C. Smith.** 2008. Measurements of copepod predation on nauplii using qPCR of the cytochrome oxidase I gene. Mar. Biol. **153:**699–707.
9. **Eiler, A., M. Johansson, and S. Bertilsson.** 2006. Environmental influences on *Vibrio* populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). Appl. Environ. Microbiol. **72:**6004–6011.
10. **Elwood, H. J., G. J. Olsen, and M. L. Sogin.** 1985. The small subunit ribosomal RNA sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. Mol. Biol. Evol. **2:**399–410.
11. **Galluzzi, L., A. Penna, E. Bertozzini, M. Vila, E. Garcés, and M. Magnani.** 2004. Development of real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Appl. Environ. Microbiol. **70:**1199–1206.
12. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
13. **Godhe, A., S. K. Otta, A.-S. Rehnstam-Holm, I. Karunasagar, and I. Karunasagar.** 2001. Polymerase chain reaction in detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from southwest India. Mar. Biotechnol. **3:**152–162.
14. **Graham, L. E., and L. W. Wilcox.** 2000. Algae. Prentice-Hall Inc., Upper Saddle River, NJ.
15. **Hare, C. E., K. Leblanc, G. R. DiTullio, R. M. Kudela, Y. Zhang, P. A. Lee, S. Riseman, and D. A. Hutchins.** 2007. Consequences of increased temperature and CO_2 for phytoplankton community structure in the Bering Sea. Mar. Ecol. Prog. Ser. **352:**9–16.
16. **Jeong, H. J., and M. I. Latz.** 1994. Growth and grazing rates of the heterotrophic dinoflagellates *Protoperidinium* spp. on red tide dinoflagellates. Mar. Ecol. Prog. Ser. **106:**173–185.
17. **John, D. E., S. S. Patterson, and J. H. Paul.** 2007. Phytoplankton-group specific quantitative polymerase chain reaction assays for RuBisCO mRNA transcripts in seawater. Mar. Biotechnol. **9:**747–759.
18. **Jouenne, F., S. Lefebvre, B. Veron, and Y. Lagadeuc.** 2007. Phytoplankton community structure and primary production in small intertidal estuarine-bay ecosystem (eastern English Channel, France). Mar. Biol. **151:**805–825.
19. **Kamykowski, D., and S.-J. Zentara.** 2003. Can phytoplankton community structure be inferred from satellite-derived sea surface temperature anomalies calculated relative to nitrate depletion temperatures? Remote Sensing Environ. **86:**444–457.
20. **Kleiboeker, S. B.** 2003. Applications of competitor RNA in diagnostic reverse transcription-PCR. J. Clin. Microbiol. **41:**2055–2061.
21. **Lassus, P., M. Bardouil, B. Beliaeff, P. Masselin, M. Naviner, and P. Truquet.** 1999. Effect of a continuous supply of the toxic dinoflagellate *Alexandrium minutum* Halim on the feeding behavior of the Pacific oyster (*Crassostrea gigas* Thunberg). J. Shellfish Res. **18:**211–216.
22. **Lin, S., H. Zuang, Y. Hou, L. Miranda, and D. Bhattacharya.** 2006. Development of dinoflagellate-oriented PCR primer set leads to detection of picoplanktonic dinoflagellates from Long Island Sound. Appl. Environ. Microbiol. **72:**5626–5630.
23. **Lindahl, O.** 1986. A dividable hose for phytoplankton sampling. ICES C.M. 1986/L:26, annex 3 (mimeo). International Council for the Exploration of the Sea, Copenhagen, Denmark.
24. **Lindahl, O., A. Belgrano, L. Davidsson, and B. Hernroth.** 1998. Primary production, climatic oscillation, and physio-chemical processes: the Gullmar Fjord time-series data set (1985–1996). ICES J. Mar. Sci. **55:**723–729.
25. **Loram, J. E., N. Boonham, P. O'Toole, H. G. Trapido-Rosenthal, and A. E. Douglas.** 2007. Molecular quantification of symbiotic dinoflagellate algae of the genus *Symbiodinium*. Biol. Bull. **212:**259–268.
26. **McAlice, B. J.** 1971. Phytoplankton sampling with the Sedgewick-Rafter cell. Limnol. Oceanogr. **16:**19–28.
27. **McQuoid, M. R., and A. Godhe.** 2004. Recruitment of coastal planktonic diatoms from benthic versus pelagic cells: variation in bloom development and species composition. Limnol. Oceanogr. **49:**1123–1133.
28. **Medlin, L. K., H. J. Elwood, S. Stickel, and M. L. Sogin.** 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **17:**491–499.
29. **Menden-Deuer, S., and E. J. Lessard.** 2000. Carbon to volume relationship for dinoflagellates, diatoms, and other protist plankton. Limnol. Oceanogr. **45:**569–579.
30. **Miller, D. N., J. E. Bryant, E. L. Madsen, and W. C. Ghiorse.** 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl. Environ. Microbiol. **65:**4715–4724.
31. **Moorthi, S. D., P. D. Countway, B. A. Stauffer, and D. A. Caron.** 2006. Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. Microb. Ecol. **52:**136–150.
32. **Müller-Navarra, D., M. T. Brett, S. Park, S. Chandra, A. P. Ballantyne, E. Zorita, and C. R. Golman.** 2004. Unsaturated fatty acid content in seston and tropho-dynamic coupling in lakes. Nature **427:**69–72.
33. **Naviner, M., J. P. Berge, P. Durand, and H. Le Bris.** 1999. Antibacterial activity of the marine diatom *Skeletonema costatum* against aquacultural pathogens. Aquaculture **174:**15–24.
34. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
35. **Prokopowich, C. D., T. R. Gregory, and T. J. Crease.** 2003. The correlation between rDNA copy number and genome size in eukaryotes. Genome **46:** 48–50.
36. **Refardt, D., and D. Ebert.** 2006. Quantitative PCR to detect, discriminate and quantify intracellular parasite in their host: an example from three microsporidians in *Daphnia*. Parasitology **113:**11–18.
37. **Round, F. E., R. Crawford, and D. G. Mann.** 1990. The diatoms. Cambridge University Press, Cambridge, United Kingdom.
38. **Roussel, Y., M. Wilks, A. Harris, C. Mein, and S. Tabaqchali.** 2005. Evaluation of DNA extraction methods from mouse stomachs for the quantification of *H. pylori* by real-time PCR. J. Microbiol. Methods **62:**71–81.
39. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* S. Krawetz and S. Misener (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ.
40. **Rynearson, T. A., J. A. Newton, and E. V. Armbrust.** 2006. Spring bloom development, genetic variation, and population succession in the planktonic diatom *Ditylum brightwelli*. Limnol. Oceanogr. **51:**1249–1261.
41. **Saravanan, V., and A. Godhe.** Physiologic differentiation and genetic heterogeneity among seasonally separated clones of *Skeletonema marinoi* (Bacillariophyceae) in Gullmar Fjord, Sweden. Eur. J. Phycol., in press.
42. **Smayda, T. J.** 1978. From phytoplankters to biomass, p. 273–279. *In* A. Sournia (ed.), Phytoplankton manual. UNESCO, Paris, France.
43. **Sun, J., and D. Liu.** 2003. Geometric models for calculating cell biovolume and surface area for phytoplankton. J. Plankton Res. **25:**1331–1346.
44. **Thomsen, H. A. (ed.).** 1992. Plankton i de inre danske farvande, vol. 11. Dansk Miljöstyrelse, Copenhagen, Denmark. (In Danish.)
45. **Throndsen, J., G. R. Hasle, and K. Tangen.** 2003. Norsk kystplanktonflora. Almater Forlag, Oslo, Norway. (In Norwegian.)
46. **Tomas, C. R.** 1997. Identifying marine phytoplankton. Academic Press, San Diego, CA.
47. **Utermohl, H.** 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Mitt. Int. Ver. Limnol. **9:**1–38.
48. **Vargas, C. A., K. Tonnesson, A. Sell, M. Maar, E. F. Moller, T. Zervoudaki, A. Giannakourou, E. Christou, S. Satapoomin, J. K. Petersen, T. G. Nielsen, and P. Tiselius.** 2002. Importance of copepods versus appendicularians in vertical carbon fluxes in a Swedish fjord. Mar. Ecol. Prog. Ser. **241:**125–138.
49. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic studies. J. Bacteriol. **173:**697–703.
50. **Zhu, F., R. Massana, F. Not, D. Marie, and D. Vaulot.** 2005. Mapping of picoeucaryotes in the marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol. Ecol. **52:**79–92.